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The Precipitation of Toroidal Collagen Fibrils

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The morphology of aggregates of calf-skin tropocollagen, precipitated by continuous injection into neutral phosphate buffers at 35°, has been studied by electron microscopy. Although most of the collagen is precipitated as normal native fibrils, a small proportion forms closed toroidal structures having the usual native band–interband pattern. Theoretical considerations, based on elastic energies in a general microfibril model, predict that the toroids should have a simple superhelical structure, and this is not inconsistent with the observations. From the theoretical energies it was possible to estimate a crude lower limit of 3kcal./mole for the free energy of association of the tropocollagen macromolecules.

The growth of collagen fibrils *in vivo* is thought to proceed by the extracellular precipitation of macromolecules of tropocollagen. The macromolecules, synthesized within the fibroblasts, are secreted into the extracellular spaces, where they aggregate on to existing fibrils or may nucleate fresh ones (Ross, 1968).

The tropocollagen molecule in solution behaves as a semi-rigid rod roughly 2800Å long and about 14Å in diameter (Boedtker & Doty, 1956; Rice, Casassa, Kerwin & Maser, 1964). The native fibril, as observed by electron microscopy and low-angle X-ray diffraction, shows a fundamental periodicity D, normally about 640Å in the dry state (for reviews see Ramachandran, 1968; Hodge, 1968). This structure may be described in terms of bundles of thin filaments or microfibrils, a few molecules in diameter, in which the macromolecules are aligned along the fibril axis (Olsen, 1963*a*,*b*; Veis, Anesey & Mussell, 1967; Smith, 1968). Within a microfibril molecules are stacked in a staggered array with a spacing D, whereas adjacent microfibrils may be staggered by integral multiples of the periodicity D (Hodge & Petruska, 1963; Smith, 1968; J. A. Chapman, unpublished work), giving the fibril its overall periodic appearance. Each molecule spans 4·4 periods and there are gaps of $0{\cdot}6D$ between the ends of longitudinally adjacent molecules (Hodge & Petruska, 1963).

Much experimental effort has gone into the simulation of fibrillogenesis *in vitro* by the precipitation of extracted tropocollagens under a variety of conditions (for a review see Wood, 1964). Typical experiments have involved the precipitation of fixed amounts of collagen induced by abrupt changes in solvent environment or temperature. The present investigation was concerned with the morphology of fibrils produced in a continuous precipitation system in which tropocollagen is injected at a fixed rate into an environment suitable for fibril formation. This approximation to a steady-state system more closely parallels the process *in vivo* and should lead to a closer understanding of the mechanisms involved.

MATERIALS AND METHODS

Collagen solutions. Tropocollagen was extracted from the fresh dermis of a 1-week-old calf by essentially standard techniques (Gross & Kirk, 1958; Bensusan & Hoyt, 1958; Rice *et al.* 1964), in the cold (0–5°). Exhaustive extraction of the tissue with neutral 1M-NaCl solution containing thiomersal (Merthiolate; 0·01g./100ml.) as antibacterial agent continued for about a week, and was followed by four successive 2-day extractions with 0·2M-acetic acid. Pooled acid-extracted fractions, salted out with solid NaCl to 20% (w/v), were redissolved in 1% (v/v) acetic acid. Dialysis against 1% acetic acid was followed by centrifugation at $80000g_{av.}$ for 1hr. The resulting solutions had a reduced viscosity of 10·8dl./g. (measured at 25° in a Cannon–Fenske capillary viscometer with a flow time for water of 280sec.), and sedimented as a single hypersharp boundary with a sedimentation coefficient (uncorrected) of 1·2s at a concentration of 0·09g./100ml. at 4° in 1% acetic acid. Protein concentrations were estimated by micro-Kjeldahl determination of nitrogen, the nitrogen content of soluble collagen being taken as 17·7% (Bowes, Elliott & Moss, 1955). Collagen solutions were used immediately or stored in frozen solution at −20° until use. Repeated freezing and thawing of solutions was avoided.

Continuous precipitation. Tropocollagen in dilute solution (typically 0·01g./100ml. in 0·005% acetic acid) was precipitated by continuous injection into neutral potassium phosphate buffer at about 35°. Injection was by peristaltic pumping through fine-bore silicone rubber tubing at a

rate of 0·22 ml./hr., with a small magnetic stirrer to ensure rapid mixing in the buffer. In all cases the initial buffer volumes were 50 ml. Standard potassium phosphate or potassium phosphate–KCl buffers were used at pH 7·1 and I 0·2–0·25 with the addition of thiomersal (0·01 g./100 ml.) to inhibit bacterial attack. On occasions, to minimize the change in pH and I as collagen solution was added, the mixture was continuously dialysed against a large volume of the buffer; for the present morphological studies this procedure did not affect the observations. The initiation of precipitation could be accelerated by pre-injection with small amounts of tropocollagen.

Control experiments included the precipitation of fixed quantities of collagen in the same buffers, both with and without continuous stirring.

Electron microscopy. Small samples of the precipitating mixtures were evaporated on carbon-stabilized collodion films supported on copper grids. Specimens were washed free of buffer salts, negatively stained with sodium phosphotungstate at pH 7·2, and examined in the Siemens Emiskop 1A or A.E.I. EM6B electron microscope.

RESULTS

The unstirred control precipitations gave suspensions of typical native collagen fibrils (Plate 1*a*). Both in the continuous-injection experiments and in stirred controls of comparable collagen concentration (about 0·001 g./100 ml.) precipitation was evident within a few hours. Electron microscopy showed the bulk of the material to consist of normal native fibrils, mostly in small thick entanglements, which in continuous injection grew larger with time, presumably by further accretion at the surface of the clump. In addition there occurred a small fraction of apparently disrupted fibrils (Plate 1*b*), probably attributable to shearing forces induced in the system by stirring.

In the continuously injected collagen system there also appeared numbers of short thin flexible fibrils and an additional fraction having a very characteristic morphology. This fraction, at a crude estimate between 0·1 and 1% of the injected tropocollagen, consisted of closed rings ('toroids') of collagen (Plates 1*c*, 2, 3 and 4) having the normal band–interband pattern characteristic of native collagen. In good specimens the band pattern continued around the loop and exhibited the usual polarized intraperiodic band pattern (Olsen, 1963*a*,*b*). The dimensions of the toroids varied in any one experiment, with circumferences in the range 10 000–100 000 Å and fibril widths 200–3000 Å, although a few thinner and apparently unstriated toroids were seen. Dimensions measured on dried specimens can give only a rough measure of the state in solution because of the disruptive effects of surface tension in the drying process (Anderson, 1956). Flattening of fibrils was apparent in many cases.

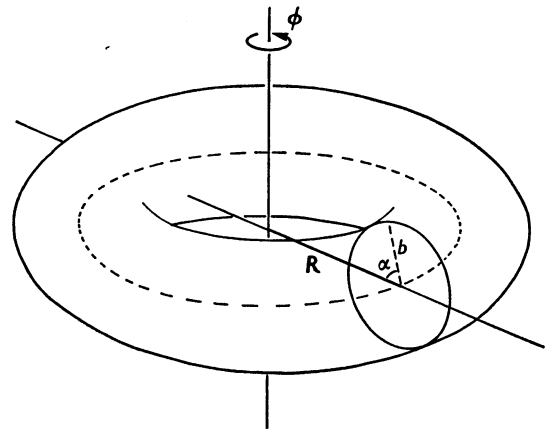


Fig. 1. Toroidal co-ordinate system. The radius of curvature of the toroid is R, and ϕ denotes the angular position about the curvature axis. Positions with respect to the fibril axis (broken line ellipse) are denoted by the off-axis distance b and the angle α.

Some of the larger toroidal forms had prominently thinner, partially disordered, regions of their circumference (Plate 1*c*), which may be interpreted as the point at which joining of the two ends of a fibril has occurred. Toroids were commonly found in association with normal fibrils (Plate 4*b* and 4*c*), either as closed loops at the end of a fibril or attached to the side of a fibril. In such cases the band–interband pattern of the toroid was in register with that on the fibril.

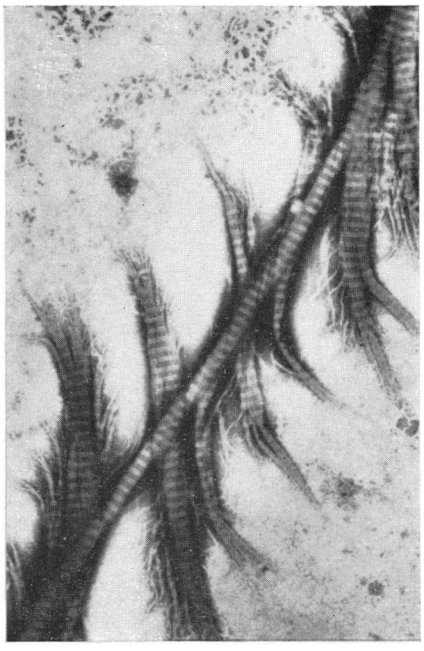
Possible structure for toroids. Considerations of symmetry in a simple microfibril model lead to general conclusions about the possible spatial configurations of the microfibrils in a toroidal fibril. Assuming, for simplicity, that the fibril is strictly toroidal, i.e. uniformly circular in cross-section, the configuration of a microfibril may be specified by using the four parameters R, b, α and ϕ (Fig. 1). The radius of curvature, R, of the fibril axis is identical for all the microfibrils in the toroid, and the distance, b, from the fibril axis is assumed to be constant for any one microfibril.

The equation $\alpha = \alpha_i(\phi)$ describes the path of the ith microfibril on the toroidal surface of constant b. Rotational symmetry about the fibril axis and translational invariance around the circles of constant α require that:

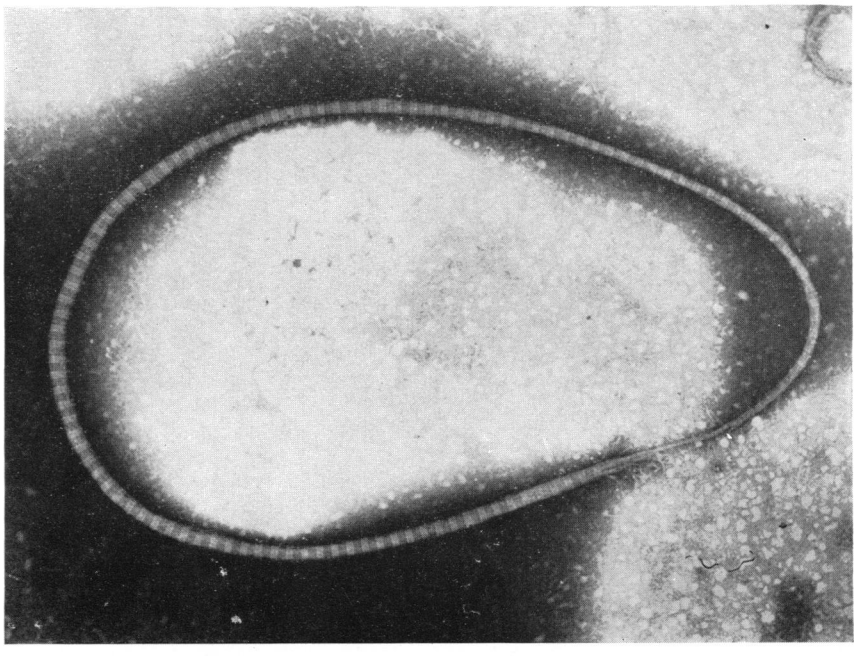
$$\alpha_i(\phi + 2\pi) = \alpha_i(\phi) + 2\pi n \qquad (n = 0, \pm 1, \pm 2, \ldots\ldots) \quad (1)$$

This is the condition that a microfibril joins up with its own end (the justification for this assump-

(*a*)



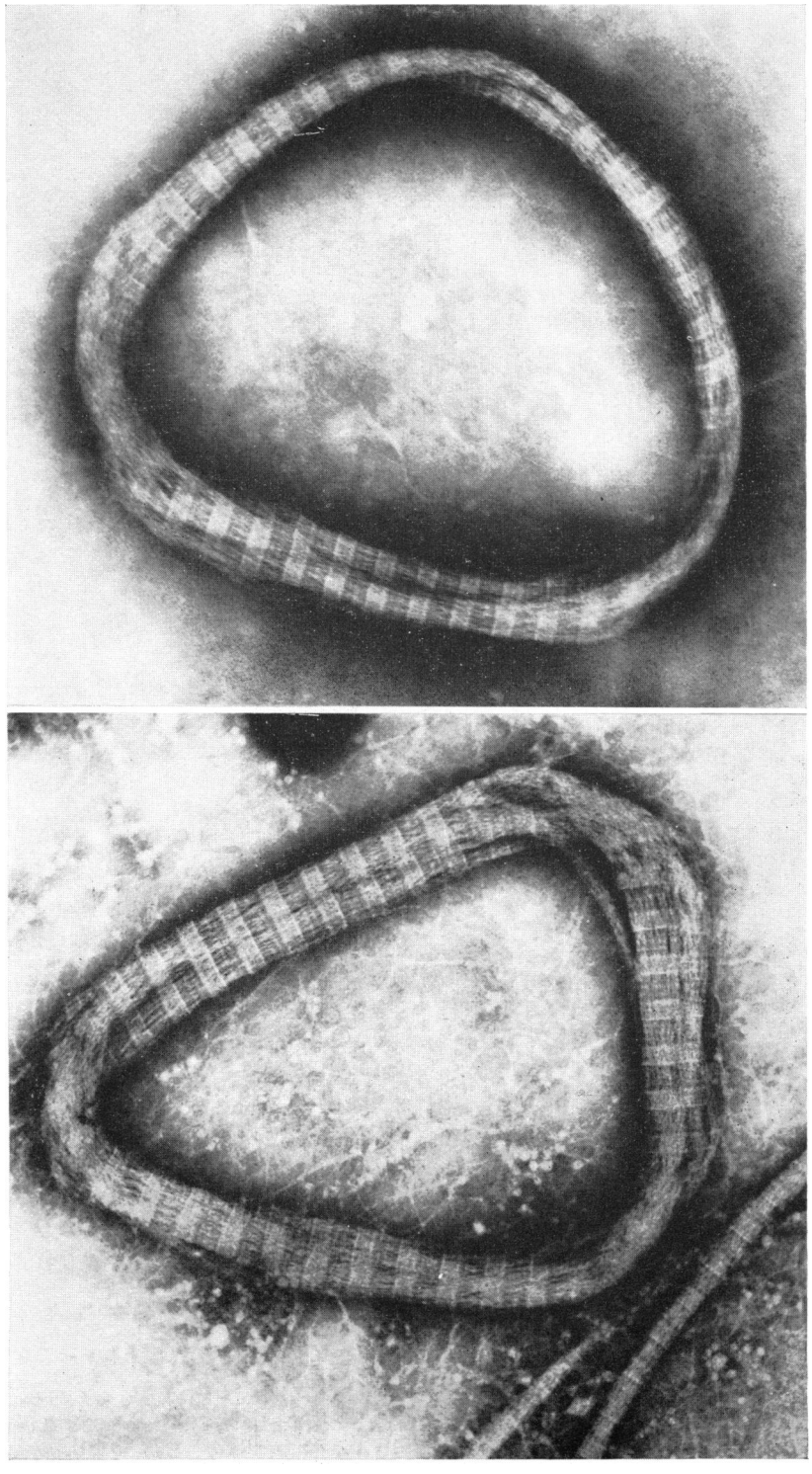
(*b*)



(*c*)

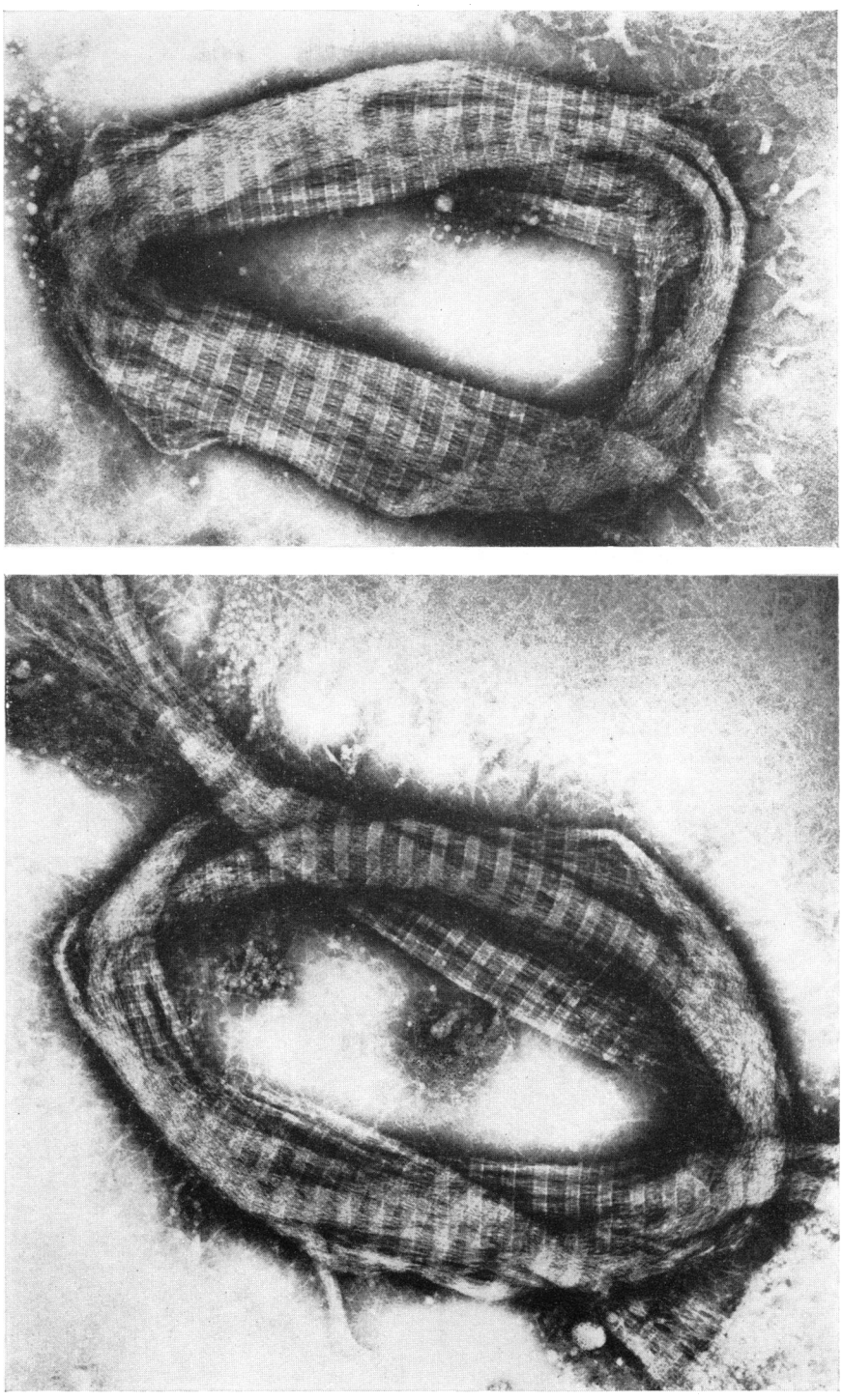
EXPLANATION OF PLATE 1

Electron micrographs of: (*a*) normal native fibrils from an unstirred control precipitation (magnification × 40000); (*b*) apparently disrupted native fibril produced during precipitation with stirring (magnification × 20000); (*c*) large toroidal fibril (magnification × 30000); the thin disordered region where the fibril ends probably join should be noted.



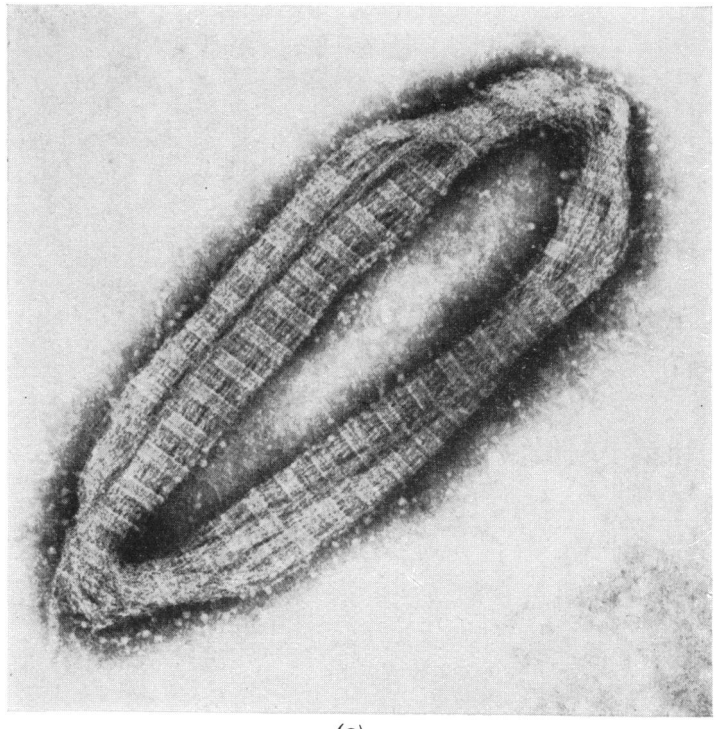
EXPLANATION OF PLATE 2

Electron micrograph of toroidal fibrils (magnification × 100000).

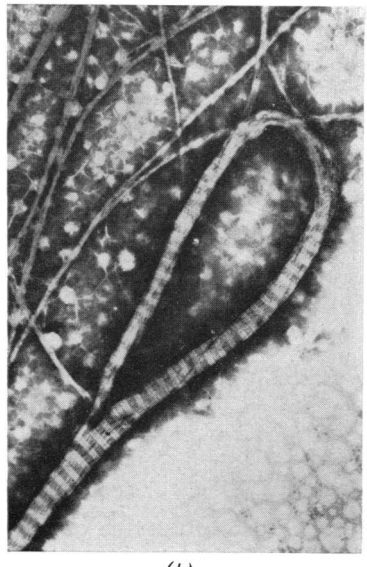


EXPLANATION OF PLATE 3

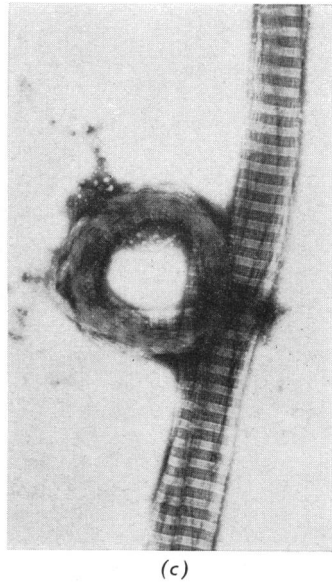
Electron micrograph of wide toroidal fibrils, apparently disrupted during drying (magnification × 80000).



(*a*)



(*b*)



(*c*)

EXPLANATION OF PLATE 4

Electron micrographs of (*a*) partially disrupted toroid (magnification × 80000); (*b*) closed loop on the end of a normal fibril (magnification × 30000); (*c*) toroid attached to the side of a normal fibril (magnification × 40000).

tion is considered below). The simplest non-trivial solution of eqn. (1) is:

$$\alpha_i(\phi) = \beta_i + n\phi \qquad (2)$$

where β_i is the angular co-ordinate of the microfibril at an arbitrary origin of the co-ordinate, ϕ.

The configurations represented by eqn. (2) are a set of superhelices in which the microfibrils coil round the fibril axis, with n turns of the helix around the toroid.

The length of a microfibril following a path given by eqn. (2) is:

$$l_{i,n} = \int_0^{2\pi} \{b^2n^2 + [R - b.\cos(\beta_i + n\phi)]^2\}^{\frac{1}{2}}.\mathrm{d}\phi \qquad (3)$$

Except for $n=0$ this expression is not soluble analytically, but an approximate solution is possible by a power series expansion of the integrand, remembering $b/R \ll 1$:

$$n = 0: \quad l_{i,o} = 2\pi(R - b.\cos\beta_i)$$

$$n \neq 0: \quad l_{i,n} \simeq 2\pi R\left[1 + \frac{b^2n^2}{2R^2} + \mathrm{O}\left(\frac{b^3}{R^3}\right)\right]$$

Assuming that the toroid is formed by joining the ends of a fibril (of original length $2\pi R$ and radius b_0) the individual microfibrils must stretch to take up the new conformation. For simple linear elastic deformation the energy required is proportional to the square of the extension. The minimum energy configuration will be that for which the mean square microfibril extension is lowest. If we define the microfibril extension as:

$$\Delta_{i,n} = l_{i,n} - 2\pi R$$

and assume a uniform distribution of microfibrils the mean square extension $\langle\Delta_n^2\rangle$ is obtained by integration over the toroid. Thus:

$$n = 0: \quad \langle\Delta_0^2\rangle = \pi^2 b_0^2$$

$$n \neq 0: \quad \langle\Delta_n^2\rangle \simeq \frac{\pi^2 n^4 b_0^4}{3R^2}$$

Since $b_0/R < 1$ the mean square extension is lowest for the $n = \pm 1$ configurations. The mean elastic energy/molecule is:

$$E_n = \frac{Kla}{8\pi^2 R^2}.\langle\Delta_n^2\rangle$$

where K is the longitudinal molecular elasticity and l and a are the length and cross-section area of the molecule respectively.

For an estimate of the elasticity the value reported for tendon is taken. The elasticity of tendon varies with age, extension, hydration and degree of cross-linking of the tissue, but for fully extended wet tendon it is about 10^{10}dynes/cm^2. (Harkness, 1961). The length, l, is about 2800 Å and the molecular cross-section area is approximately 150 Å^2. From micrographs it was estimated that $b_0/R \leqslant 0{\cdot}14$.

With these parameters the mean elastic energy for the $n = \pm 1$ configuration is less than about 1 kcal./mole of tropocollagen (cf. $kT \simeq 0{\cdot}6$ kcal./mole). For $n = 0$ the energy is about 200 times as great, and for other n values the energies rise by the factors n^4.

The symmetry arguments were based on the continuity of structure around the toroid. This is not necessarily true, since the junction of the fibril ends could well form a natural discontinuity, which in a superhelical description would allow for non-integral n values in eqn. (2). In this case the approximate integration of eqn. (3) is not valid, so the problem was approached numerically. The mean square extension for model toroids containing various numbers of superhelical microfibrils were calculated by numerical integration of eqn. (3) on the I.C.T. Atlas computer for non-integral values of n. The results were unambiguous and are illustrated in Fig. 2. In all cases the mean square extension drops steeply to a minimum at $n = \pm 1$.

Further, by using the same criterion of minimum microfibril extension, the most favourable superhelical configuration for a fibril bent into a series of arcs approaching a complete toroid was calculated. For a fibril bent round, until its ends almost meet, the minimum extension is for $n = \pm 1$ superhelix as before.

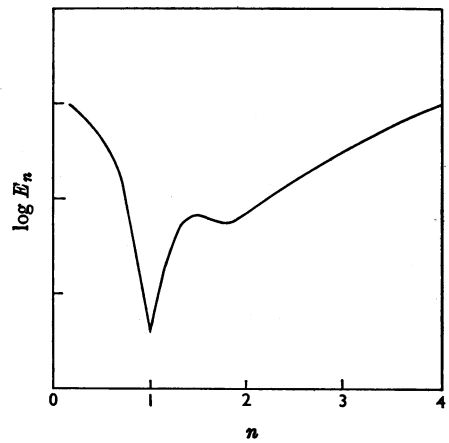


Fig. 2. Semi-logarithmic plot of the mean elastic energy/molecule, E_n (in arbitrary units) as a function of the helical parameter, n, obtained by numerical integration of eqn. (3) for a model toroid with $b_0/R = 0{\cdot}1$.

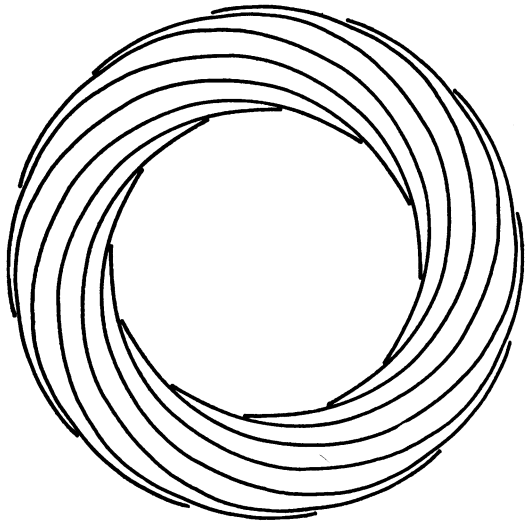


Fig. 3. Microfibril arrangement in a $n = \pm 1$ superhelical toroid, seen in plane projection along the axis of the toroid.

Thus, provided that longitudinal elastic deformation of the microfibrils is the dominating influence on quaternary structure, a superhelical configuration of the $n = \pm 1$ type is preferred (Fig. 3), and the energy required to form the structure is comparable with or less than thermal energies.

I have, so far, neglected the energy due to bending of the microfibrils and distortion of the intermolecular bonds, although to some extent the latter has been taken into account by using the elasticity value for whole tendon rather than individual microfibril (which is, anyway, unknown). No information is available on the bending of rod-like macromolecules, but a crude estimate of the energy is possible by assuming that the molecule behaves as a homogeneous cylindrical rod with a microscopic elasticity equal to the macroscopic elasticity of tendon. Such a model is a gross oversimplification, but should give a reasonable estimate. For toroids of small radius this energy comes to less than 0·2kcal./mole, and is correspondingly smaller for the larger toroids. It is assumed that the bond deformation energy will be of a comparable order of magnitude, so that the total energy expended in forming a fibril into a circle will be less than about 1·5kcal./mole.

For thermodynamic stability this expended energy must be outweighed by the free energy liberated when fibril ends join, and it is thus possible to put a crude lower limit on the free energy of association ($-\Delta F$) of the molecules. Under these conditions, ignoring any possible entropy changes in bending the fibril, $-\Delta F > 3$kcal/mole.

DISCUSSION

Toroidal aggregates have previously been observed in precipitates of synthetic polypeptides from non-hydrogen-bonding solvents (Blais & Geil, 1968). Under such conditions the polypeptides have the semi-rigid α-helical conformation and aggregate in microfibrillar bundles, so the process may be qualitatively similar to that for collagen toroids.

Gross microfibrillar structure can be seen in micrographs of negatively stained toroids and the observations are not inconsistent with the superhelical structure proposed above, but an unambiguous interpretation is difficult because in such pictures we see both sides of the object superimposed (Klug & Finch, 1965). The possible superhelical arrangement is particularly evident in micrographs of toroids that appear to have partially disrupted in the drying process (Plates 3 and 4*a*). Some of the micrographs of replicas of toroids by Blais & Geil (1968) also show superhelical structure (see, in particular, their Fig. 4*e*).

The direction of twist of the helix is arbitrary in the simple model since we assume that the microfibrils initially lie parallel to the fibril axis. In view of the difficulty of determining the sense of the superhelix in negatively stained toroids shadow-casting has been attempted, but so far the resolution has not been sufficient to display much surface structure of the toroids.

The energy required to bend a fibril into a toroid depends on the ratio fibril width/curvature, b_0/R, but is less than about $2kT$/molecule and usually is considerably less. This value was obtained by using the maximum observed b_0/R ratio and ignoring the possibility that b_0 may be increased after formation of the toroid by further accretion at the surface. The lower limit of 3kcal./mole for the aggregation free energy cannot be considered as more than an order to magnitude estimate, but seems in reasonable accord with values observed in comparable systems involving non-covalent specific aggregation. Thus for the polymerization of tobacco-mosaic-virus sub-unit protein $-\Delta F = 4{\cdot}56$kcal./mole (Banerjee & Lauffer, 1966). For antibody–antigen complexes the free-energy changes are in the range 5–10kcal./mole, and for the dimerization of insulin $-\Delta F = 4{\cdot}93$kcal./mole (Waugh, 1959). Caspar (1966) has estimated that for macromolecular aggregates to be stable at physiological concentrations the interaction free energies should be in the range 4–10kcal./mole.

The formation of toroidal fibrils may be visualized as follows. Once nucleated, the aggregates develop into fibrils by preferential accretion at the ends. As they grow the fibrils will bend and flex under stirring and thermal fluctuations. Occasionally

the fibril ends will approach one another and, if the approach is sufficiently close, there is a finite chance that the ends will join and the joint be secured by the rapid accretion of more collagen. The likelihood of a close approach of two ends requires the fibril to be fairly short, but not so short and thick as to require much more than thermal energies to bend it. To stabilize the toroid there must be sufficient free collagen available in solution to precipitate and reinforce the join: this is presumably why toroids were observed only in the continuous-injection experiments, since under normal conditions precipitation is so fast that the toroids do not have time to form. Blais & Geil (1968) did not employ continuous injection, but in their system precipitation was comparatively slow.

For long fibrils the chance of two ends meeting is small, but there seems no reason why an end may not curl round and attach to the side of the fibril (Plates 4*b* and 4*c*). In general, however, the longer fibrils tend to form entanglements in which flexion of the fibril is hindered by the surrounding fibril matrix. Topological constraint by the surrounding tissue is possibly why toroidal fibrils have not been observed *in vivo*, though this is, of course, a much more complex environment than the one studied.

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